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Communication to the Editor

Ciclesonide Inhibits SARS-CoV-2 Papain-Like Protease *in Vitro*

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The emergence of coronavirus disease 2019 (COVID-19), a novel identified pneumonia resulting from the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, has significantly impacted and posed significant challenges to human society. The papain-like protease (PLpro) found in the nonstructural protein 3 of SARS-CoV-2 plays a vital role in viral replication. Moreover, PLpro disrupts the host immune response by cleaving ubiquitin and interferon-stimulated gene 15 from host proteins. Consequently, PLpro has emerged as a promising drug target against SARS-CoV-2 infection. Computational studies have reported that ciclesonide can bind to SARS-CoV-2 PLpro. However, the inhibitory effects of ciclenoside on the PLpro have not been experimentally evaluated. Here, we evaluated the inhibitory effects of synthetic glucocorticoids (sGCs), including ciclesonide, on SARS-CoV-2 PLpro *in vitro* assay. Ciclesonide significantly inhibited the enzymatic activity of PLpro, compared with other sGCs and its IC_{50} was 18.4 ± 1.89 μM. These findings provide insights into the development of PLpro inhibitors.

Key words ciclesonide, coronavirus disease 2019 (COVID-19), papain-like protease (PLpro), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

INTRODUCTION

The emergence of coronavirus disease 2019 (COVID-19), a novel coronavirus pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has deeply affected and presented substantial challenges to human society. SARS-CoV-2 features an enveloped single-stranded positive-sense RNA genome belonging to the beta coronavirus genus.[1] The RNA genome encodes two large overlapping polyproteins, PP-1a and PP-1ab.[2] Viral cysteine proteases, namely the main protease (Mpro) and papain-like protease (PLpro), cleave polyproteins into 16 nonstructural proteins (Nsps), each of which plays a crucial role in viral replication. PLpro cleaves polyproteins containing a consensus sequence LXGG↓X and is capable of cleaving the viral polyprotein to generate Nsp1, Nsp2, and Nsp3, which are essential for virus replication.[2] Additionally, PLpro removes ubiquitin and the ubiquitin-like interferon-stimulated gene 15 (*ISG15*) from cellular proteins, inhibiting interferon-α/β signaling. The deubiquitination and deISGylation activities of PLpro lead to dysregulation of host inflammation and evasion of the innate immune response.[3] Thus, PLpro is considered a promising therapeutic target owing to its essential role in cleaving and disrupting host responses. Recently, based on molecular dynamics simulations, ciclesonide has been reported as a prospective compound for the allosteric inhibition of PLpro.[4] Allosteric inhibition affects the substrate-binding ability and *ISG15* affinity of PLpro.[4] Ciclesonide, a synthetic glucocorticoid (sGC), is an approved antiasthmatic drug with a substantial global market. sGCs, including dexamethasone, are recommended for preventing severe diseases in both adults and children with respiratory illnesses.[5,6] However, the inhibitory effects of ciclesonide on PLpro have not been experimentally evaluated. In this study, we purified SARS-CoV-2 PLpro and examined its potential inhibitory activity of sGCs, including ciclesonide, through *in vitro* assays.

MATERIALS AND METHODS

Expression and Purification of SARS-CoV-2 PLpro The SARS-CoV-2 PL protease gene, which corresponds to amino acid sequence 746-1064 of NSP3 (NCBI reference sequence: YP_009725299), was optimized for bacterial expression. The coding vector (pGEX-6p-1-SARS-CoV-2-PLpro) with N-terminal GST (Genescript, Piscataway, NJ, U.S.A.) was transformed into *Escherichia* (*E.*) *coli* BL21 (DE3) cells and *E. coli* were grown at 37 °C until OD 600 nm was reached 0.6. Expression was induced by the addition of 1 mM isopropyl ß-D-1-thiogalactopyranoside and *E. coli* were incubated for 16 h at 20 °C. The *E. coli* culture was subsequently centrifuged at 3000 × ***g*** for 10 min at 4 °C, washed with phosphate buffered saline, and the resultant pellet was re-suspended in lysis buffer (25 mM Tris–HCl, pH 7.5, with 0.5% Triton X-100). The lysates were homogenized by sonication, and the supernatant were obtained to be centrifuged at 13000 × ***g*** for 10 min at 4 °C. PLpro was isolated using GST-accept (Nacalai, Kyoto, Japan) and was subsequently ultrafiltered. PLpro was further treated with PreScission Protease (Cytiva, Tokyo, Japan) to remove GST, and purified using GST-accept.

***In Vitro* PL Protease Activity** Ciclesonide was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dexamethasone (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), prednisolone (FUJIFILM Wako Pure Chemical Corporation), and betamethasone (Tokyo Chemical Industry Co., Ltd.) were dissolved in 10% dimethyl sulfoxide (DMSO) and used for this assay. The PLpro assays were conducted in a total volume of 100 μL, comprising the following components: 25 mM Tris–HCl pH 7.5, 10 mM DTT, 150 mM NaCl, 20 nM SARS-CoV-2 PLpro, and the compound. After 5 min of incubation, PLpro activities were measured with the addition of Z-arginine (Arg)-leucine (Leu)-Arg-glycine (Gly)-Gly-MCA at a final concentration of 20 μM. Fluorescent signals (excitation, 360 nm; emission, 460 nm) were acquired every 3 min for 15 min using a Synergy H1 instrument (Agilent Technologies, Santa Clara, CA, U.S.A.). All experiments were conducted in triplicate, and the average values (mean ± standard deviation)

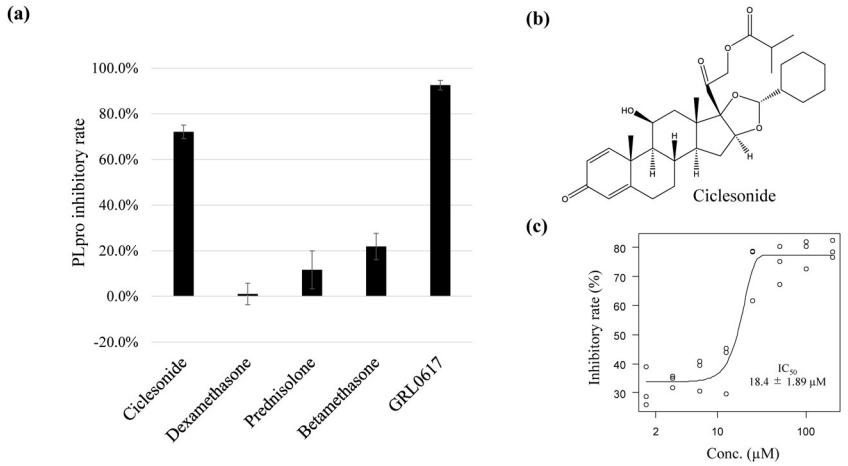

Fig. 1. PLpro Inhibition of Ciclesonide (a) Inhibitory Effects of Synthetic Glucocorticoids (Ciclesonide, Dexamethasone, Prednisolone, and Betamethasone; 50 μM) on PLpro Activity

GRL0617 (50 μM) was used as positive control. Vertical bars indicate the standard deviation ($n = 3$). (b) Structural formula of ciclesonide. (c) IC_{50} curve of ciclesonide.

were determined.

RESULTS AND DISCUSSION

In the present study, PLpro was purified from *E. coli* and used for an *in vitro* assay. Using the purified PLpro, we first evaluated the expression of GRL0617, a PLpro inhibitor. The IC_{50} of GRL0617 was 3.70 ± 0.70 μM, in consistency with a previously reported value[3] (Supplementary Fig. S1). Next, we evaluated the PLpro inhibitory activity of sGCs, including ciclesonide, dexamethasone, prednisolone, and betamethasone (Fig. 1a). Among these compounds, ciclesonide ($72.1 \pm 2.9\%$) effectively inhibited the protease activity of PLpro at concentration of 50 μM. Moreover, dexamethasone ($1.1 \pm 4.7\%$), prednisolone ($11.6 \pm 8.3\%$), and betamethasone ($21.6 \pm 5.8\%$) showed a little or no inhibition of PLpro. Furthermore, ciclesonide exhibited dose-dependent inhibition, with an IC_{50} is 18.4 ± 1.89 μM (Figs. 1b, c).

Ciclesonide, an inhaled steroid agent, is a safe anti-inflammatory drug used to treat asthma, and its anti-inflammatory effect is likely attributable to the inhibition of PAK1.[5] *In silico* studies have shown that ciclesonide inhibits viral replication by binding directly to the Nsp15 endonuclease of SARS-CoV-2.[7,8] Ciclesonide's allosteric inhibition of PLpro shrinks the active site pocket of PLpro using computational techniques and surface area, which might affect substrate binding ability.[4] In the present study, we demonstrated the inhibitory effects of ciclesonide on PLpro activity using an *in vitro* assay.

PLpro inhibition by ciclesonide would lead to the development of new COVID-19 treatments. Although many research groups have conducted extensive high-throughput screenings to discover PLpro inhibitors, there are currently not approved PLpro inhibitors for clinical use. As shown in Fig. 1a, ciclesonide significantly inhibited PLpro activity, whereas dexamethasone, prednisolone, and betamethasone had no or weak inhibitory effects on PLpro activity. Focusing on the structure of ciclesonide, it has characteristic structures with a 2-cyclohexyl-1,3-dioxlane moiety attached to the cyclopentane ring of the steroidal core and 2-methyl propanoic acid ester. The structure is not possessed by the other three sGCs. Our results demonstrate the potential to develop novel PLpro inhibitors that enhance the structural features of ciclesonide.

In conclusion, we examined the inhibitory effects of synthetic glucocorticoids on SARS-CoV-2 PLpro by using an *in vitro* assay. We demonstrated that ciclesonide significantly inhibits PLpro activity. Novel PLpro inhibitors are expected to be developed by enhancing the structural features of ciclesonide.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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